In the Specification (*continued*):

Please insert the new detailed description of the invention as follows (this should occupy page 11, line 1 to page 22, line 5 of the existing specification). This detailed description of the invention consists of material from the original submission, but re-arranged. The bulk of the original was canceled when a substitute specification was submitted with the 11/23/2004 amendment. Thus, this is a restoration of canceled matter and is presented as a new insertion (MPEP § 608.01(s)). Therefore, a marked-up version is not presented, other than the completely struck out version on the previous pages. The origin of all text in this new detailed description of the invention, as to where it was in the original submission, is indicated in the remarks section of this amendment. This detailed description of the invention is presented as clean on the following pages:

DETAILED DESCRIPITION OF THE INVENTION:

The object of the present invention is to provide a method for the sensitive non-radioactive assessment of ligand binding to insoluble surfaces. Specifically, the method developed measures the binding of transferrin, concanavalin-A, avidin, annexin-V, and insulin to cell surfaces. The basic detailed method using fluorescein-conjugated transferrin as a detectable antibody-recognizable hapten tracer follows. Specific alterations of this procedure for other ligands follow.

Fluorescein-conjugated iron-saturated (holo) human transferrin (FITC-Tf) was obtained from commercial sources. Cultured cells to be measured were grown to 50 – 60 % confluence in 12 well plates. Cells were incubated with serum-free minimal essential media (alpha modification; ∝-MEM) for 12 h and then again with fresh ∝-MEM for another 12 h. The cell number in three wells was determined by trypsinization of those cells followed by enumeration on a cell counter. Media in remaining wells was replaced with 1 ml binding buffer (BB) which consisted of: 25 mM HEPES in ∝-MEM containing 2mg/ml of bovine serum albumin (BSA); pH 7.5. The cell wells were then allowed to equilibrate to 4°C in a refrigerator. Sets of 5 replicate wells received increasing amounts of FITC-Tf, from 0.06 to 2.0 μg/ml final FITC-Tf. Two wells of each FITC-Tf concentration set then received unlabeled holo human transferrin so that the final [Tf] = 100 μg/ml. After a 2h incubation at 4°C, all media was saved (= unbound samples), and the wells were all washed 4 times by the addition and drainage of 1 ml of 4°C PBS. All wells then received 0.5 ml of an RIPA cell lysing solution which consisted of PBS

containing 1% v/v NP-40, 0.5% v/v deoxycholic acid, 0.1% v/v SDS, 100 μg/ml phenylmethyl sulfonyl chloride, and 0.1 TIU/ml Aprotinin. Cells were incubated with the lysing solution for 30 min at 4°C and all lysates were pipetted into separate 1.5 ml conical tubes. The tubes were centrifuged at 5,000 X g for 10 min and 400 uL of each supernatant was transferred to a fresh tube. All of these tubes received 166 uL of a 4X concentrate SDS-PAGE treatment solution, and were treated at 95°C for 10 minutes.

Treated samples were loaded onto a 12 X 12 cm 10% acrylamide SDS-PAGE electrophoresis gel (150 uL/sample) and electrophoresed at 40 mA constant current until the dye front was 1 cm from the bottom of the gel. The gel was equilibrated in a transfer buffer of 48 mM Tris, 39 mM glycine. A 14 X 14 cm nitrocellulose membrane was equilibrated in transfer buffer and the gel and membrane assembled into a transfer apparatus and immersed in transfer buffer. Gel components were transferred to the membrane at a constant voltage of 40 V for 1.5h.

The membrane was blocked at 4°C overnight in a block solution consisting of Tris buffered saline (TBS: 25 mM Tris, 0.15 M NaCl, pH 7.8) containing 0.1% tween 20 and 5% w/v non-fat dry milk. The membrane was incubated with 1:1000 rabbit anti-FITC in block solution for 2h at 25°C, and washed three times (20 min each) with 50 ml TBS. The membrane was incubated with 1:2000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG for 2h at 25°C and washed again. Each membrane was covered with

an enhanced chemiluminescence (ECL) substrate for HRP, was wrapped in plastic, and was loaded into an X-ray film cassette along with an 8 X 10 inch piece of photographic film. The film was developed after 1 min exposure and an additional film was added which was developed after 20 min exposure. Bands produced on the film were quantified using an imager.

Unbound samples are run similarly to cell lysate samples. Typically, these have to be diluted 1:10 – 1:100 in SDS-PAGE treatment solution prior to electrophoresis, to produce a signal within a readable range. The assay is standardized by loading known amounts of pure FITC-Tf onto an electrophoresis gel and repeating all of the above procedures. The signal returned from the imager is plotted against the amount of FITC-Tf contained in the band and a standard curve is constructed to calculate the amounts of FITC-Tf bound by the cells.

A schematic of the strategy of the assay is shown in Figure 1. A cell monolayer is exposed to a solution of FITC-Tf (Figure1A) or FITC-Tf plus an excess of unlabeled Tf (Figure1B). In either case, 3 molecules of Tf bind per cell. When washed and lysed (Figure 1C), cells from Figure1A produced a lysate containing 9 molecules of FITC-TF (Figure 1E) whereas cells from Figure1B produce a lysate containing 1 molecule of FITC-Tf (Figure 1D, Figure 1F). In Figure 1H, both samples are electrophoresed and the gels blotted (Figure 1I). With the cells from Figure1A, 9 molecules of FITC-Tf are present in the 70,000 molecular weight region of the blot; when this blot is incubated

with rabbit anti-FITC and then with goat anti-rabbit-IgG-HRP, a large band is seen (Figure 1J, Figure 1K, Figure 1L). With the cells from Figure1B, only one molecule of FITC-Tf is present on the blot and a minimal band is seen on the blot after ECL (Figure 1J, Figure 1K, Figure 1L).

Reproductions of actual enhanced chemiluminescence films obtained when this assay was performed are shown in Figure 2. Figure 2A shows the measurement of FITC-transferrin (FITC-Tf) binding to MTLn2/TfR cells. Cells at 50 – 60 % confluency growing in 12 well plates were serum-starved, then treated at 4° C with increasing levels of FITC-Tf. After 2h, cells were washed, lysed, and equal cell equivalents were electrophoresed, blotted, incubated with goat anti-FITC, then with anti-goat-HRP and an HRP ECL substrate. The blot was then analyzed using an imager. Figure 2A shows results from lysates from cell exposed to the concentration of FITC-TF listed above the blot. Figure 2B (left side) shows results from lysates from cells treated as in Figure 2A, but also with 100 μg/ml of unconjugated Tf. Figure 2C shows results from a blot treated as in Figure 2A but loaded with pure FITC-TF standards in the amounts (in ηg) indicated on the top. The pure FITC-Tf samples were electrophoresed, blotted, and measured using the two antibodies mentioned in Figure 2A, followed by ECL. Figure 2D shows results from the quantification of Figure 2C using an imager, indicating the type of standard curves achievable.

The binding of annexin V to cell surfaces has been recognized as an indicator of early apoptosis (Zhang *et. al.*, 1997). With conventional procedures, cells are removed from plates, treated with FITC-annexin V, and analyzed by FACS. The removal of cells from tissue culture plates using conventional trypsin or EDTA reagents can in itself induce cell stress, apoptosis, and cellular annexin V binding (Darzynkiewicz *et. al.*, 1998; LeGall *et. al.*, 2000). Therefore, the conventional use of annexin V binding as a measure of apoptosis in adherent cells is problematic. In contrast, this invention would measure the binding of FITC-annexin V to adherent cultured cells *in situ* (Figures 3 and 4), where binding and washing occur first, before the cells are removed from plates for analysis. Therefore, the amount of FITC-annexin V detected would accurately represent that bound by cells in their natural culture environment. Thus, the method outlined in this invention circumvents conventional problems and provides for a more authentic measure of natural cellular annexin V binding.

A schematic of the strategy of the assay when used to detect apoptotic cells is shown in Figure 3. Cells in early apoptosis are known to bind the protein Annexin V whereas normal cells bind little or none of this protein. Cells in apoptosis (Figure 3A) or normal non-apoptotic cells (Figure 3B) are exposed to a solution of FITC-Annexin V. When washed (Figure 3C, Figure 3D) and lysed (Figure 3E, Figure 3F), cells from Figure 3A produced a lysate containing FITC-Annexin V (Figure 3E) whereas cells from Figure 3B produce a lysate containing no FITC-Annexin V (Figure 3F). In Figure 3G and Figure 3H, both samples are electrophoresed and the gels electro-blotted (Figure 3I). With the

cells from Figure 3A, the FITC-Annexin V molecules are present in the 33,000 molecular weight region of the blot. When this blot is incubated with anti-FITC and then with anti-goat-IgG-HRP, HRP is localized to the 33Kd region of the blot (Figure 3J, Figure 3K) and the HRP-containing bands are detected on photographic film using an HRP chemiluminesent substrate (Figure 3L, Figure 3M). This produces a band on the film at 33 Kd (Figure 3M). With the cells from Figure 3B, no FITC-Annexin V is present on the blot (Figure 3I), the initial antibody and therefore the second antibody do not bind (Figure 3J, Figure 3K), no light is produced upon incubation with an HRP chemiluminesent substrate (Figure 3L, Figure 3M), and no band is seen on the film (Figure 3M).

Figure 4 shows the results obtained when this assay was used to measure the binding of FITC-Annexin V to rat MTLn3 mammary adenocarcinoma cells, as described in Figure 3. The cells were grown to confluence in six well plates. Cells were induced to apoptose by treatment with 4 ug/ml Camptothecin (dissolved in DMSO). Controls received DMSO only. After 24h, wells were washed three times with and equilibrated in 1 ml binding buffer (25 mM HEPES, .15 M NaCl, 2.5 mM $CaCl_2$, pH 7.5). FITC-Annexin V was added to 50 ηg/ml and the cells were incubated for 30 min at 25° C. Cells were then washed extensively with binding buffer, and lysed in 1 ml of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min., and the supernatants were separated by SDS-PAGE. Also run on the same gel were increasing levels of pure FITC-Annexin V. Separated proteins were blotted onto a nitrocellulose membrane which was

blocked and then incubated with rabbit anti-FITC and then goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL. A scan of the photographic film is shown in Figure 4A. Results of the quantification of the standards is shown in Figure 4B. The curve from Figure 4B was used to calculate Annexin V bound by the cells, the results of which are shown in Figure 4C. The results indicate greater Annexin V binding by the camptothecin treated cells.

Figure 5 shows the results obtained when this assay was used to measure the binding of FITC-Concanavalin A (Con A) to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. The growth media was replaced with a binding buffer consisting of 25 mM HEPES buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Con A was added to replicate wells so that the final concentrations of FITC-Con A were 0.1, 1.0, and 10.0 μg/ml. One well of each FITC-Con A concentration also received 200 μg/ml of native (un-conjugated Con A). The cells were incubated for 2h at 4° C, washed extensively with PBS, and lysed in 800 μL of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min., and the supernatants were separated by SDS-PAGE. Also run on the same gel were increasing levels of pure FITC-Con A. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL. A scan of the film is shown in Figure 5A. Results of quantification of the standards is shown in Figure 5B. The curve from Figure 5B was used to calculate

specific Con A bound by the cells, the results of which are shown in Figure 5C.

Figure 6 shows results obtained when the samples from Figure 5 were analyzed by a dot blot procedure. For the standards, increasing volumes (2, 4, 8, and 16 μL) of a 100 η g/ml FITC-Con A solution were applied to a nitrocellulose membrane. For the lysates, 4 μL of lysates from cells treated with 0.1, 0.5, and 1.0 μg/ml FITC Con A (with or without an excess native Con A) were applied to the membrane. The membrane was blocked, incubated with rabbit anti-FITC, then with goat anti-rabbit IgG-HRP, and HRP-containing sites detected with ECL (Figure 6A, Figure 6B). The dots were quantified using an imager. Data from the standards (Figure 6A, Figure 6C) were used to determine the amount of Con A bound by the cells (Figure 6B, Figure 6D). This displays the usefulness of the technique in a dot-blot procedure, where the SDS-PAGE and electro-blotting steps are eliminated.

Figure 7 shows results obtained when this assay was used to measure the binding of FITC-Avidin to rat MTLn3 mammary adenocarcinoma cells. The cells were grown to confluence in six well plates. The growth media was replaced with a binding buffer consisting of 25 mM HEPES buffered MEM containing 3 mg/ml liquid gelatin (as a carrier and blocking protein), at pH 7.5. The cultures were taken to 4° C and FITC-Avidin was added to replicate wells so that the final concentrations of FITC-Avidin were 0.1, 0.2, and 0.4 μg/ml. The cells were incubated for 2h at 4° C, washed extensively with PBS, and lysed in 1 ml of RIPA lysing solution. The lysates were centrifuged at 5,000 X g for 5 min., and the supernatants were separated by SDS-PAGE. Also run on the same

gel were increasing levels of pure FITC-Avidin. Separated proteins were blotted onto a nitrocellulose membrane which was blocked and incubated with rabbit anti-FITC and then goat anti-rabbit IgG-HRP. HRP containing bands were detected by ECL. A scan of the photographic film is shown in Figure 7A. Results of quantification of the standards is shown in Figure 7B. The curve from Figure 7B was used to calculate specific Avidin bound by the cells, the results of which are shown in Figure 7C.

Figure 8 shows the results obtained when this assay was used to measure the binding of FITC-Insulin to human K562 erythroleukemia cells. Logarithmically growing cells in suspension culture were collected by centrifugation and washed twice by suspension in and centrifugation from a binding buffer consisting of alpha-MEM containing 5 mg/ml BSA and 25 mM HEPES (pH 7.5). Cells were adjusted to a density of 2 X 10^6/ml (in binding buffer), and were equilibrated to 4° C. To 1 ml of cell suspension was added 20 µl of 1mg/ml FITC-Insulin (in binding buffer; final concentration = 20 µg/ml). An additional tube also received non-conjugated Insulin at a level of 200 µg/ml. Cell suspensions were incubated for 2h at 4° C while rotating slowly, and the cells were collected and washed three times by suspension in and centrifugation from binding buffer. Cell pellets were lysed in 0.4 ml/tube of Schager-Von Jagow (SVJ) electrophoresis system treatment solution and treated at 95° C for 5 min. Lysates (100 µl each) were separated by SDS-PAGE run according to Schager-Von Jagow along with FITC-Insulin standards, and treated aliquots of the cell-unbound incubation mixture. Gel components were transferred to nitrocellulose and membrane-associated FITC detected

as described with other ligands. In Figure 8, a scan of the ECL film is shown. Increasing signal is returned for increasing loads of FITC-insulin in the standards (Figure 8, Lanes 1-3). The FITC-insulin bound by the cells is easily observed (Figure 8, Lane 4), and this is reduced significantly when excess un-conjugated insulin was present (Figure 8, Lane 5). This procedure consistently displays higher molecular weight forms of insulin formed after application to cells, perhaps due to the presence of insulin binding proteins (Figure 8, Lanes 4-7).

The assay could be used to verify the hybridization of biotin-labeled DNA to other DNA molecules. In one scenario, biotin-labeled PCR products are hybridized to an immobilized DNA probe which specifically recognizes the desired PCR product (among a mixture of non-specific products). After binding, the bound PCR product is released through heat de-naturation, is separated by agarose electrophoresis, electro-blotted to nytran, and is detected by incubation with species-x anti-biotin followed by incubation with anti-species-x IgG-HRP and ECL. The final result yields a major band at the expected bp size of the PCR product. Any non-specific bands of different size can be ignored during analysis of the film by an image analyzer. As with the above stated protein procedures, the proper molecular weight of the desired product is verified.

The replacement of electrophoresis with dot-blot techniques is possible. This would require that the only immune-recognizable conjugated component present prior to dot-blotting would be the desired product and/or absolutely minimal interaction of either

antibody with non-specific sample components

General applications: The assay strategy can apply to any ligand conjugated with a compound which can be specifically recognized by an antibody. In particular, anti-digoxygenin, anti-rhodamine and anti-biotin antibodies exist which would recognize ligands conjugated with those compounds. The material to which the ligand binds to can be other than cells. Any particles or other insoluble material can serve as the binding surface. Centrifugation and re-suspension of suspended particulate binding substrates would serve as a method for washing those of unbound ligand. The current method requires that the bound ligand be removed from the binding surface so that it can be separated by electrophoresis. It must also bind to a conventional transfer membrane for detection with the antibody. Other specific applications accomplished to date include the study of the binding of FITC-conjugated concanavalin A to cells, the study of the binding of Avidin to cells, and the study of the binding of Annexin-V to cells. With the latter protein, this assay could be utilized to assess cellular apoptosis without the need for a FACS analyzer.

Conclusion: the invention is a procedure for measuring the binding of an entity (ligand) to a surface by using a hapten-conjugated version of the ligand (hapten-ligand), where the hapten is recognizable by an antibody. An excess of the hapten-ligand is presented to the binding surface and excess (unbound) hapten-ligand is washed off. Bound hapten-ligand is then solubilized (removed) and applied to a membrane support or separated by electrophoresis and applied to a membrane support. The membrane-bound

hapten-ligand is detected by application of an enzyme-conjugated antibody to the hapten; or by application of an antibody to the hapten followed by application of an enzyme-conjugated antibody to the anti-hapten antibody. The resultant membrane-associated enzyme is detected and quantified by the application of a color or light-producing substrate which reacts with the enzyme. This assay method has the advantages of providing verification of the molecular weight of the binding substance (ligand) via the electrophoresis step. It eliminates the need for radioactive materials. The procedure provides for high sensitivity detection as the dual antibody incubation steps amplify the signal significantly. The procedure allows for easy standardization as different user-definable levels of a standard solution of the Hapten-ligand can be simultaneously applied to the electrophoresis gel or to the dot-blot or slot-blot membrane.